Induction of Epidermolysis Bullosa Acquisita in Mice by Passive Transfer of Autoantibodies from Patients

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Epidermolysis bullosa acquisita (EBA) is an autoimmune sub-epidermal blistering disease characterized by autoantibodies to type VII (anchoring fibril) collagen. To date, however, direct evidence for a pathogenic role of human EBA autoantibodies has not been demonstrated. In this study, we affinity-purified anti-type VII collagen antibodies from EBA patients' sera and then injected them into adult hairless immunocompetent mice. Mice injected with EBA autoantibodies developed skin fragility, blisters, erosions, and nail loss on their paws – all features of EBA patients. By clinical, histological, immunological, and ultrastructural parameters, the induced lesions were reminiscent of human EBA. Histology showed bullous lesions with an epidermal–dermal separation. IgG and C3 deposits were observed at the epidermal–dermal junction. All mice had serum antibodies that labeled the dermal side of salt-split human skin like EBA sera. Direct immunogold electron microscopy specifically localized deposits of human IgG to anchoring fibrils. (Fab')$_2$ fragments generated from EBA autoantibodies did not induce disease. We conclude that EBA human patient autoantibodies cause sub-epidermal blisters and induce EBA skin lesions in mice. These passive transfer studies demonstrate that human EBA autoantibodies are pathogenic. This novel EBA mouse model can be used to further investigate EBA autoimmunity and to develop possible therapies.

INTRODUCTION

Epidermolysis bullosa acquisita (EBA) is an acquired auto-immune blistering disease of the skin (Roenigk et al., 1971). EBA patients have autoantibodies in their blood and skin directed against structures in the skin called anchoring fibrils (Woodley et al., 1984, 1986, 1988). Anchoring fibrils are located at the dermal–epidermal junction (DEJ) of skin and function to adhere the epidermal layer of the skin onto the dermal layer (Briggaman and Wheeler, 1975; Sakai et al., 1986; Burgeson, 1993). Although the precise role of autoantibodies to type VII collagen in the pathogenesis of EBA remains to be established, it is very likely that anchoring fibrils are critical for the epidermal layer of skin to adhere to the dermal layer. Evidence for this is that patients who are born with a defect in the gene that encodes for type VII collagen have a paucity of normal anchoring fibrils and exhibit skin fragility and skin blisters just like EBA patients (Uitto and Christiano, 1992, 1994).

Anchoring fibrils, wheat-stack-shaped structures in the skin that hold the epidermis and dermis together, are composed of type VII collagen (Sakai et al., 1986; Keene et al., 1987). This collagen is composed of three identical alpha chains, each consisting of a 145 kDa central collagen-ous triple-helical segment characterized by repeating Gly-X-Y amino-acid sequences, flanked by a large 145 kDa amino-terminal, non-collagenous domain (NC1), and a smaller 34 kDa carboxy-terminal non-collagenous domain (NC2) (Burgeson, 1993). We and others have shown that the NC1 domain of type VII collagen is highly antigenic and that most EBA autoantibodies are directed against sub-domains within NC1 (Gammon et al., 1993; Lapiere et al., 1993; Jones et al., 1995).

The etiology of EBA is unknown. Nevertheless, there is some evidence to suggest that antibodies to type VII collagen may be pathogenic and able to cause epidermal–dermal disadherence. Recently, we immunized rabbits and raised a high titer antiserum to the NC1 domain of human type VII collagen. We injected this antibody into hairless immuno-competent mice and the mice developed a skin condition that had many of the features of EBA in humans (Woodley et al., 2005). Another recent study by Sitaru et al. (2005) showed that the injection of rabbit polyclonal antibodies to the NC1 domain of mouse type VII collagen into adult nude,

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BALB/c, and C57BL/6 mice also induced sub-epidermal skin blisters that were reminiscent of human EBA.

Although these studies with antibodies raised in rabbits against human or mouse type VII collagen are suggestive and interesting, they do not truly meet the autoimmune disease equivalent of ''Koch's Postulate'', which is to demonstrate that the human patient's autoantibody can duplicate the disease in an animal (Witebsky, 1966; Rose and Bona, 1993). In this regard, all previous attempts to develop an animal model using EBA patients' autoantibodies transferred passively into animals were unsuccessful (Shigemoto et al., 1988; Chen et al., 1992; Borradori et al., 1995).

In this report, we affinity purified anti-NC1 autoantibodies from EBA patients' sera and injected the antibodies into adult immunocompetent, hairless mice. We found that the animals developed a sub-epidermal bullous disease with clinical, histological, immunological, and ultrastructural features similar to human EBA. These results provide evidence that human EBA autoantibodies to the NC1 domain of type VII collagen are pathogenic and capable of inducing epidermal–dermal separation of skin.

RESULTS

Characteristics of affinity-purified EBA IgG fractions

We affinity-purified antibodies to the NC1 domain of type VII collagen from the sera of two EBA patients who were plasmaphoresed for therapeutic purposes. The antigenic specificity of the anti-type VII collagen antibodies was

Figure 1. **Characterization of affinity-purified EBA antibodies.** (**a**) The figure shows the specificity of affinity-purified EBA autoantibodies for the NC1 domain of type VII collagen. Purified recombinant NC1 as well as other extracellular matrix components (400 ng/well) were separated on a 6% SDS-PAGE gel and transferred to nitrocellulose membranes. The transferred proteins were incubated with affinity-purified EBA antibodies at a dilution of 1:2,000 and horseradish peroxidase conjugated anti-human IgG (1:5,000) followed by ECL detection. Lanes 1, 2, 3, 4, 5, and 6 are NC1, type I collagen, type IV collagen, fibronectin, laminin-1, and laminin-5, respectively. The location of the 145 kDa recombinant NC1 and molecular weight markers are indicated. (**b–d**) The figure shows immunolabeling of mouse and human skin with the affinity-purified EBA antibodies. Immunofluorescence staining was performed on (**b**) human skin, (**c**) mouse skin, and (**d**) salt-split human skin. The tissue was labeled with EBA antibodies diluted 1:1,000 and an FITC-conjugated goat anti human IgG. Note that the affinity-purified EBA antibody strongly labeled the BMZ of both mouse and human skin and the dermal floor of salt-split human skin. d, dermis; e, epidermis. Bars, 200 μM.

characterized by immunoblot analysis as shown in Figure 1a. The antibodies only labeled the 145 kDa NC1 domain of type VII collagen (lane 1) and did not label other matrix proteins including type I collagen, type IV collagen, fibronectin, laminin-1, and laminin-5 (Figure 1a, lanes 2–6).

To determine whether the purified antibodies reacted with murine skin, we performed indirect immunofluorescence (IIF) analysis on both human and murine skin. As shown in Figure 1b, the antibodies strongly labeled the basement membrane zone (BMZ) of both human and mouse skin at dilution titers over 1:50,000. The control IgG fraction purified from normal human sera did not label either mouse or human skin (data not shown). IIF testing against salt-split human skin substrate revealed that the antibody bound to the dermal side of the salt-split skin, consistent with labeling-type VII (anchoring fibril) collagen and characteristic of EBA serum (Gammon et al., 1984, 1990).

Clinical evaluation of mice injected with EBA antibodies

SKH1 mice were given daily intradermal injections of affinity-purified anti-type VII collagen antibodies from EBA sera or control normal human sera at doses from 20 to 100 μg/g body weight/per day. The characteristics of the EBA anti-type VII collagen antibodies are outlined in Table 1. As shown in Figure 2, animals injected with the EBA anti-type VII collagen antibodies ($n=40$) showed numerous blisters, erosions, and crusts forming from ruptured blisters as early as day four in the middle back near the injection sites (Figure 2a). With time, additional lesions were observed on the body (Figure 2b and c), ears, and paws of the animals (Figure 2d) with subsequent injections. Nail loss was observed in 70% of the injected mice (Figure 2e and f). Mice continued to develop new widespread lesions even 6 weeks after the initial series of injections (Figure 2c). As summarized in Table 1, cutaneous lesions occurred in 36 of 40 experimental mice given EBA antibodies. In contrast, none of the 10 animals that received identical doses of control IgG injections developed any cutaneous lesions. Furthermore, five animals injected
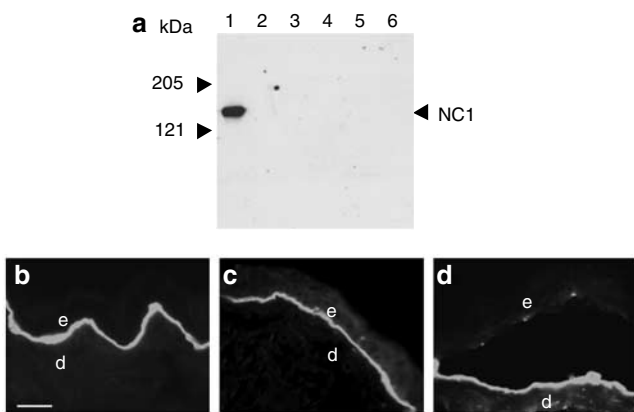
Table 1. Characterization of the affinity-purified EBA antibodies used and the incidence of disease in injected mice

Source of IgG	IIF titer[1]	Dose of IgG injected μg/g of body weight/day	No. of mice with skin lesions[2]
Patient 1	50,000	20–100	19/20
Patient 2	50,000	20–100	17/20
Control IgG[3]	0	20–400	0/10
Flow-through IgG[4]	0	20–400	0/5

[1]Titers of concentrated affinity-purified EBA antibodies tested against normal mouse skin.
[2]The numerator denotes the number of mice in which skin lesions developed; the denominator denotes the total number of mice that received injections.
[3]From pooled normal human serum.
[4]Flow-through IgG were from flow-through fractions of NC1 affinity column that were depleted the reactivity to the NC1 domain.
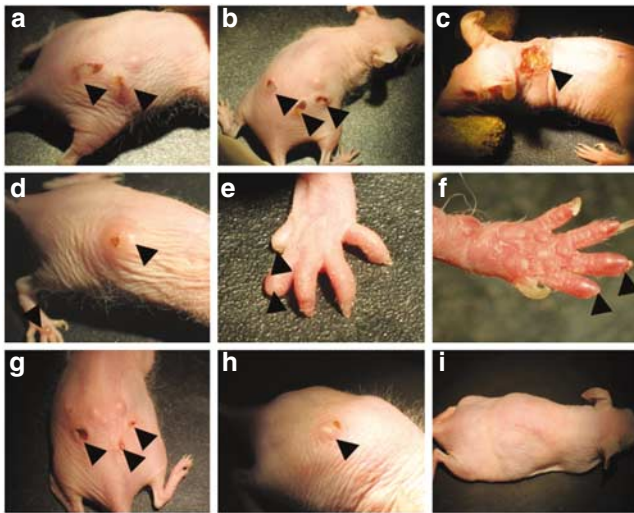
Figure 2. **Clinical appearance of SKH1 mice injected with EBA antibodies.** (**a–f**) Middle back of SKH1 mice were injected intradermally with EBA antibodies at 50 μg/g body weight once every day for 8 consecutive days. Four to six days after the injections were started, animals developed numerous blisters, erosions, and crusts forming from ruptured blisters (**a**, 4 × and **b**, 6 ×). Lesions were observed on the animals' paws (**d**) at 10 days and neck (**c**) at 6 weeks after the initial injections (8 ×). Nail loss was also observed in some mice (**e** and **f**). (**g–i**) Relation between the dose of EBA antibodies injected into mice and the extent of cutaneous lesions induced in the animals. The mice shown in panel g, panel h, and panel i received 50, 10, or 2.5 μg IgG/g body weight, respectively, daily for 8 consecutive days. The arrows point to areas of blisters and erosions induced by EBA antibodies 8 days after the initial injections (8 ×). No lesions were observed in the animal in panel i.

Table 2. Titers of affinity-purified EBA antibodies correlates with the extent of disease in mice

Number of mice	Dose of IgG injected μg/g of body weight/day	Extent of disease[1]	IIF titer[2]
1	50	++	1,000
2	50	++	400
3	50	++	800
4	50	++	1,000
5	10	+	100
6	10	+	80
7	10	+	100
8	10	+	40
9	2.5	−	—
10	2.5	−	—
11	2.5	−	—
12	2.5	−	—

[1]Extent of disease was scored as follows: −, no lesions; +, fewer than three lesions; ++, more than three lesions and measured at 10 days after initial injection.
[2]Titers of circulating IgG from injected mice tested against normal mouse skin.
Disease activity and IIF titers were determined after mice were injected with IgG dose as indicated above each day for 8 days.

with EBA IgG depleted of reactivity to the NC1 domain of type VII collagen (flow-through fractions from NC1 affinity column) did not develop blisters.

Given the nature of our experimental protocol, it is impossible to know the exact kinetics of a single injection inducing a given lesion. This is because we injected animals every single day for 8 days. If we observed a blister or multiple blisters arising on day 4, for example, we can not tell if that blister arose because of the injection at any particular day.

We examined the correlation between the extent of clinical skin lesions and the dose of IgG injected by studying 12 additional animals. As shown in Figure 2g–i, mice injected with high doses of EBA antibodies (>50 μg/g body weight/per day (n=4)) developed more lesions (++) than animals injected with lower doses of EBA antibodies (10 μg/g body weight/per day (n=4)) (+). Animals injected with only 2.5 μg/g body weight (n=4) of EBA antibodies exhibited no lesions at all (−). Table 2 summarizes the results of these dose–response studies. These data demonstrate that increasing the amount of injected IgG results in an increase in the circulating titer of BMZ specific anti-type VII collagen antibody, which, in turn, was associated with increased skin lesions.

Histological and immunological examination of mice injected with EBA antibodies
Histological examination of lesional skin from mice injected with EBA antibodies showed a full sub-epidermal blister with a

clean separation between the epidermis and dermis (Figure 3a). There was also a mild to moderate scattered dermal inflammatory infiltrate consisting mostly of mononuclear cells and a few neutrophils. In contrast, mice injected with similar concentrations of control normal human IgG demonstrated a normal epidermis and dermis without any pathological alterations (Figure 3b).

Direct immunofluorescence (DIF) of perilesional and lesional skin demonstrated that all of the mice injected with EBA antibodies had human IgG deposits at the DEJ (Figure 3c). Also, in 85% (34 out of 40) of the injected mice, murine complement component C3 deposits were detected at the DEJ (Figure 3c). Within the dermal inflammatory infiltrate, neutrophils could be detected (Figure 3c). In contrast, no IgG, C3, or neutrophils were observed in the skin of mice receiving equivalent amounts of control normal human IgG.

Mice that received injections of EBA antibodies had titers of circulating antibody (1:500–1:2,000) when assayed by IIF against either normal or salt-split human skin. This titer is comparable to the starting antibody titer of crude EBA sera before purification (1:1,280–1:5,120). As shown in Figure 4, serum samples from the experimental mice had human antibodies that labeled the DEJ of normal human skin (Figure 4a) and the dermal side of the salt-split human skin (Figure 4b). In contrast, serum from mice injected with control normal human IgG did not bind to the DEJ of human skin even at dilutions of 1:10 (Figure 4c).
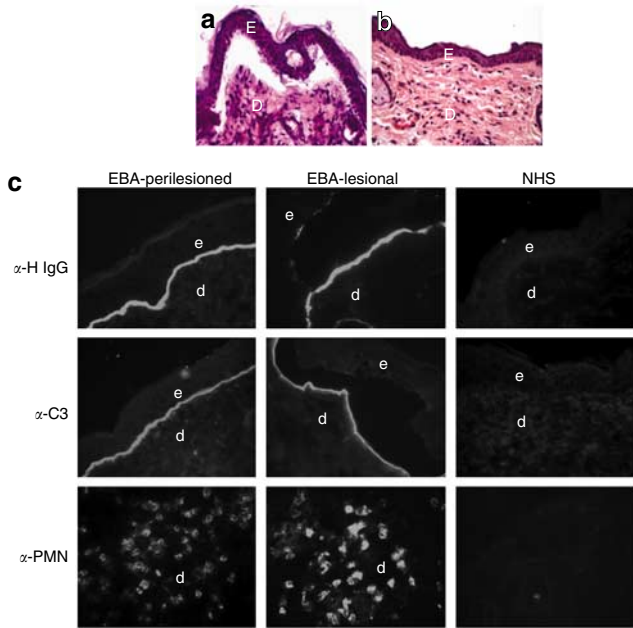
Figure 3. Histological and immunological examination of lesional skin of SKH1 mice injected with EBA antibodies. (**a** and **b**) Histological appearance of skin lesions induced by EBA autoantibodies. Hematoxylin and eosin staining of (**a**) lesional murine skin revealed separation of the epidermis (E) from the dermis (D). This histologic finding is similar to that seen in lesional skin of EBA patients. (**b**) No epidermal–dermal separation was seen in mice receiving equivalent amounts of normal human control IgG. (**c**) Immunofluorescence analysis of SKH1 mice skin injected with EBA antibodies. Cryosections of perilesional and lesional skin were labeled with FITC-conjugated goat anti-human IgG (α-H IgG), FITC-conjugated goat anti-mouse C3 (α-C3), and FITC-conjugated goat anti-mouse neutrophils (α-PMN), respectively. Note the linear deposits of human IgG and murine C3 at the BMZ of perilesional and lesional skin in mice injected with EBA antibodies. Please also note the scattered neutrophils in the dermis. In contrast, in mice receiving equivalent amounts of purified control normal human IgG (NHS), no deposits of human IgG or murine C3 were detected. d, dermis; e, epidermis. Original magnification, × 25.

Immunogold electron microscopy examination of mice injected with EBA antibodies

Direct immunogold electron microscopy of perilesional mouse skin injected with EBA antibodies showed human IgG deposits specifically localized to the lamina densa (where anchoring fibrils insert), anchoring fibrils, and anchoring plaques (Figure 5a). In lesional skin, a clear dermal–epidermal separation was present in the sub-lamina densa region (Figure 5b), similar to what is seen in human EBA blisters. In contrast, mice that received control human IgG injections had no deposits of human IgG at the DEJ of mouse skin (data not shown).

F(ab')₂ fragments of pathogenic EBA autoantibodies do not induce blisters

We wished to determine if complement activation was required for the induction of blisters and nail loss in the mice injected with human EBA autoantibodies. We prepared F(ab')₂ fragments of the affinity-purified EBA autoantibodies demonstrated to induce skin blisters by passive transfer in mice. The complement binding, Fc domains, of the
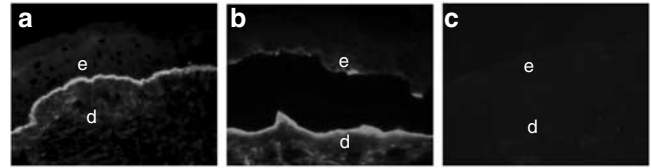

Figure 4. Immunolabeling of human skin with serum from mice injected with EBA antibodies. Sections of (**a**, **c**) normal human skin and (**b**) salt-split human skin were stained with mouse serum obtained from mice injected with either (**a**, **b**) EBA antibodies or (**c**) control human IgG at a dilution of 1:100. Note that circulating antibodies labeling the BMZ of human skin and the dermal floor of salt split human skin were found in the serum samples from mice injected with EBA antibodies, but not in those injected with normal human control IgG. d, dermis; e, epidermis.
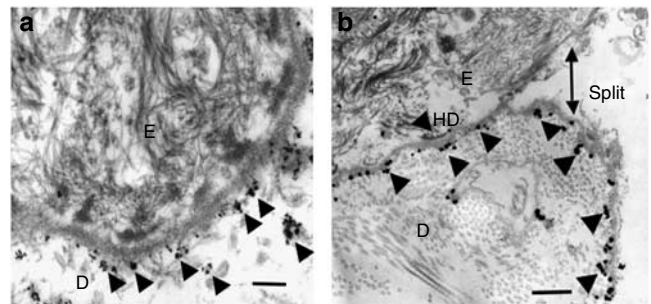

Figure 5. Immunoelectron microscopy of mice skin injected with EBA antibodies. (**a**) Perilesional and (**b**) lesional skin of mice injected with affinity-purified EBA autoantibodies were subjected to direct immunogold labeling with goat anti-human IgG conjugated with a 1 nm gold particle. Note that the injected human IgG specifically deposited along the lamina densa of the BMZ, anchoring fibrils, and anchoring plaques. A split is also present in the sub-lamina densa region of the lesional skin. Arrows denote gold particle-labeled human IgG. D, dermis; E, epidermis; and HD, hemidesmosome. Bars, for (**a**) 200 nm and for (**b**) 500 nm.

antibodies were removed. We injected F(ab')₂ fragments into SKH1 mice using equimolar doses as intact EBA IgG. In contrast to the experiments with complete EBA IgG, the skin of mice injected with F(ab')₂ did not develop clinical blisters or any lesions (Figure 6a). When the skin of the injected mice was subjected to DIF, the skin exhibited F(ab')₂ at the DEJ, but no human IgG Fc or murine complement C3 (Figure 6b–d). As expected, F(ab')₂ fragments prepared in an identical manner from control human IgG and injected into the mice did not induce lesions (data not shown).

DISCUSSION

In this study, we used human EBA patient sera to transfer passively an EBA-like disease into hairless immunocompetent mice. The human EBA sera were affinity-purified against the NC1 domain of human type VII collagen producing high-titer human anti-NC1 antibodies. When these anti-human NC1 antibodies were injected into the mice, the mice developed sub-epidermal blisters, erosions, and nail loss, reminiscent of the disease features seen in human EBA patients. By IIF testing, we showed that the mice had anti-human NC1 antibodies circulating in their blood, again, similar to many human EBA patients. By DIF on skin sites far away from the
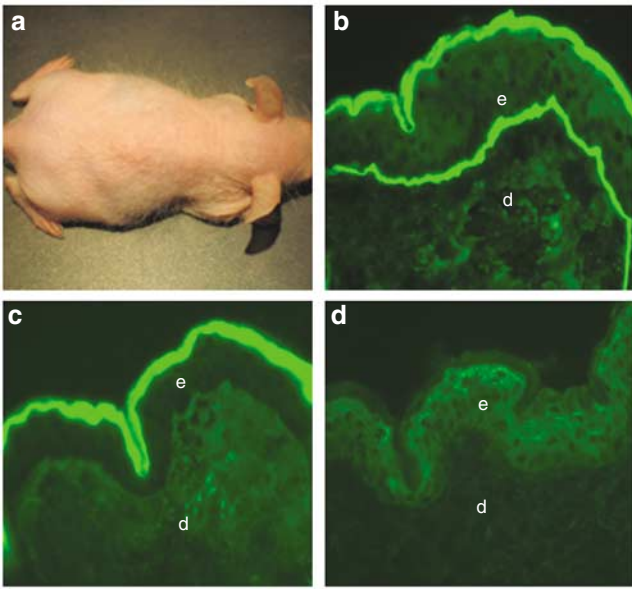
Figure 6. Clinical and immunological examination of SKH1 mice injected with the F(ab′)$_2$ fragments generated from EBA antibodies. (**a**) Clinical appearance of skin injected with F(ab′)$_2$ fragments at 40 μg/g body weight once every day for 8 consecutive days showed no signs of skin blisters. (**b**–**d**) Immunofluorescence analysis of SKH1 mice skin injected with F(ab′)$_2$ fragments of EBA antibodies. Cryosections of injected skin were labeled with FITC-conjugated (**b**) goat anti-human Fab, (**c**) goat anti-human Fc, and (**d**) goat anti-mouse C3, respectively. Note the strong binding of F(ab′)$_2$ fragments to the BMZ, but the absence of Fc and murine C3 complement within the BMZ.

injected site on the back, such as the mouse's ears, hips, or paws, we detected human IgG deposits within the BMZ of the mouse's skin (data not shown). Likewise, we found human IgG deposits at the junction of the esophageal epithelium and lamina propria when we tested the upper one-third of the animal's esophagus by DIF. A few mice exhibited weight loss after the eighth day injection period. We killed these mice and noted erosions in their oral mucosa (data not shown). Many, but not all, patients with EBA have involvement of the oral mucosa and upper one-third of the esophagus. Like human EBA patients, the injected anti-human NC1 antibodies bound to the anchoring fibrils of the animals as demonstrated by immunoelectron microscopy. Therefore, taken together, the mice injected with human EBA patient autoantibodies exhibited a blistering disease with clinical, histological, and immunological features akin to human EBA patients.

The possibility that human EBA autoantibodies are pathogenic has never before been proven, but was indirectly alluded to by two recently described EBA mouse models published by our group and another group (Sitaru et al., 2005; Woodley et al., 2005). In these studies, rabbits were immunized with type VII collagen and anti-type VII collagen rabbit IgG raised. When injected into mice, these rabbit anti-type VII collagen antibodies induced skin blisters suggestive of EBA (Sitaru et al., 2005; Woodley et al., 2005). However, it is important to recognize that these studies used immunized rabbit IgG rather than human EBA patients' sera. Therefore, these previous studies with immunized rabbit antibodies did not prove the pathogenicity of EBA patients' autoantibodies. Several

previous attempts to demonstrate directly the pathogenicity of human EBA autoantibodies by passively transferring them into mice were unsuccessful (Shigemoto et al., 1988; Chen et al., 1992; Borradori et al., 1995). There are several possible reasons why previous attempts failed. First, Borradori et al. (1995) used neonatal mice for IgG passive transfer and only two injections were given. Therefore, the mice were exposed to the antibody for a very limited incubation time. The turnover of anchoring fibril structures is thought to be very slow (Burgeson, 1993). Therefore, it is likely that the resident anchoring fibrils in the mice may have continued to function despite the presence of EBA autoantibodies. It is possible that multiple injections of human EBA autoantibodies and a longer period of time are needed to reproduce EBA in an animal. In this study, we injected adult mice every day for 8 days.

Another potential problem encountered in previous studies was that the amount of human anti-type VII collagen antibody injected was too low. In our previous study, we did not inject affinity-purified antibodies (Chen et al., 1992). Rather, we injected the whole IgG fractions from EBA sera. The actual amount of anti-type VII collagen antibody in these IgG fractions was likely extremely low. In the study by Shigemoto et al. (1988), EBA passive transfer was attempted in adult mice for three consecutive days using total IgG. However, the amount of total IgG fraction injected was only 0.13 mg/g body weight. In this study, unlike previous attempts, we enriched the actual anti-type VII collagen antibodies by affinity purification against an NC1 domain affinity column and concentrating the anti-NC1 antibody after the elution. Therefore, virtually 100% of the antibodies injected into the animals were specific for human type VII collagen. The titers of the resultant affinity-purified antibodies were extremely high (over 1:50,000 by IIF against human or mouse skin sections).

Type VII collagen is a large macromolecule with approximately half of its molecular mass consumed by the large, non-collagenous globular domain, NC1. We and others have shown that NC1 domain of type VII collagen contains four immunodominant epitopes targeted by the majority of EBA sera (Gammon et al., 1993; Lapiere et al., 1993; Jones et al., 1995). The fact that affinity-purified human EBA sera against NC1 could produce the full-blown manifestations of EBA in animals suggests that the main pathogenic epitopes of type VII collagen reside within the NC1 domain. Consistent with this notion is the fact that EBA IgG antibodies depleted of any NC1 activity (i.e. the flow through fractions from the NC1 affinity column) failed to induce any lesions in the mice.

The etiology of EBA is unknown. Anchoring fibrils are reduced in the skin of EBA patients, but the underlying mechanism leading to this reduction is unknown (Nieboer et al., 1980; Yaoita et al., 1981). One possible explanation for diminished anchoring fibrils in EBA would be that the antigen–antibody complex invokes an inflammatory response that induces destruction of type VII collagen and other BMZ components. This mechanism is perhaps predominant in the inflammatory, BP-like, variety of EBA characterized by vesiculobullous lesions on inflamed skin (Gammon et al., 1982). Sitaru et al. (2005) showed that rabbit antibodies to the

NC1 domain of murine type VII collagen triggered an inflammatory reaction with complement activation and the recruitment of leukocytes. In this EBA murine model, blister induction required the Fc portion of the rabbit IgG and activation of terminal complement components (Sitaru *et al.*, 2005). Their data suggested that inflammation might be the primary mechanism for blister formation in that model. In this study, the administration of human EBA anti-type VII collagen antibodies fixed murine complement at the DEJ. We further demonstrated that injection of mice with pepsin-derived F(ab')$_2$ fragments prepared from human EBA antibodies did not induce clinical or histological EBA lesions. These data suggest that the Fc domains of pathogenic EBA antibodies are critical for sub-epidermal blister formation. As antibody-specific activation of the complement system is mediated via the Fc portion of IgG molecules, our results suggest that anti-type VII collagen EBA antibodies induce sub-epidermal blisters in our murine model via activation of the complement system. In this regard, it is interesting to note that both of the EBA patients used in this study had inflammatory EBA and C3 deposits at their DEJ by DIF.

However, because classical EBA presents with little skin inflammation, another possible mechanism may be owing to autoantibodies perturbing the ability of type VII collagen to interact with other basement membrane components. That is, the EBA autoantibodies target certain epitopes on the NC1 domain of type VII collagen and make the function of the collagen compromised. This could perturb critical direct interactions between type VII collagen and other extracellular components within the DEJ such as type IV collagen, laminin-5, and fibronectin (Lapiere *et al.*, 1994; Chen *et al.*, 1997a, b, 1999). This mechanism may explain the skin fragility and trauma-induced blisters in patients with classical EBA who lack significant inflammation but have markedly defective epidermal–dermal adherence.

In summary, this study provides evidence that EBA autoantibodies directed against type VII collagen are pathogenic when passively transferred into hairless immuno-competent mice. Our study establishes that EBA is an example of a human autoimmune disorder that can be passively transferred from human beings to laboratory animals. This EBA experimental mouse model will be useful for dissecting the molecular and immunological mechanisms of sub-epidermal blister formation in EBA and for developing more effective therapy for EBA.

MATERIALS AND METHODS

Patients and sera

Plasma samples were collected from two patients with EBA during the early phase of their disease (before treatment). One patient was a clear bullous-pemphigoid-like EBA patient with tense blisters on inflammatory bases. Her initial IIF titer on salt-split skin was 1:320 but rose to 1:1,280 3 days before plasmaphoresis. The second patient was a woman who was misdiagnosed as bullous pemphigoid for at least 10 years, but then developed lesions on her hands, feet, sacrum, and knees that more resembled classical EBA with skin fragility, milia, and scarring in addition to more inflammatory bullous-pemphigoid-like lesions on her trunk. Her IIF titers on

salt-split skin ranged between 1:1,280 and 1:5,120, with the latter value 7 days before plasmaphoresis. These patients were plasma-phoresed for therapeutic purposes, which generated large volumes of plasma rich in anti-type VII collagen antibodies. In general, these two EBA patients had (i) an active, chronic, bullous disease; (ii) sub-epidermal blisters as assessed by routine light microscopy of lesional skin; (iii) IgG and C3 deposits detected at the DEJ by routine DIF; (iv) IgG deposits localized to the dermal floor of the patient's skin when the DEJ was fractured through the lamina lucida by treatment with 1 M NaCl (Woodley *et al.*, 1983; Gammon *et al.*, 1990); (v) immuno-reactivity to the NC1 domain of type VII collagen by ELISA and immunoblot analysis (Chen *et al.*, 1997a, b); (vi) IgG deposits detected within the sub-lamina densa region of the DEJ using direct immunoelectron microscopy (Yaoita *et al.*, 1981); and (vii) IIF on salt-split normal human skin substrate demonstrating serum IgG antibodies labeling the dermal floor of the substrate with the titers ranging from 1:1,280 to 1:5,120. The study was conducted according to the Declaration of Helsinki Principles and was approved by the University of Southern California Institutional Review Board. Patients gave their written informed consent.

Mice

SKH1 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and hosted at the University of Southern California Facility. These are hairless mice with an intact immune system. Four- to eight-week-old animals were injected with affinity-purified anti-type VII collagen antibodies from the EBA patients' sera or control IgG fractions from the sera of normal human subjects at the same IgG concentrations. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Preparation and characterization of IgG fractions

The patient's plasma was first diluted with antibody binding buffer (20 mM sodium phosphate, pH 7.0) at a 1:5 dilution and then centrifuged at 4,000 r.p.m. to remove insoluble particulate material. The supernatant were then subjected to chromatography using a protein G-Sepharose Fast Flow column following the manufacturer's recommendation (Amersham Biosciences, Uppsala, Sweden). Control IgG fractions were prepared in an identical manner from a commercial lot of human γ-globulins obtained from several hundred normal donors (Sigma, St Louis, MO). IgG fractions from EBA patients were further affinity-purified using recombinant NC1 covalently coupled cyanogen bromide-activated Sepharose 4B column following the manufacturer's instructions (Amersham Bioscience, Sweden). The 145 kDa NC1 domain of human type VII collagen was generated in human 293 cells by stable transfection with a pRC/cytomegalovirus expression vector and purified to homogeneity from conditioned media, as described previously (Chen *et al.*, 1997a, b). Affinity-purified EBA antibodies were dialyzed against phosphate-buffered saline, concentrated by Centricon Plus-20 ultrafiltration (Amicon, Lexinton, MA) to 20–50 mg/ml, filter-sterilized, and stored at −20°C. By IIF, antibody titers ranged from 1:10,000 to 1:50,000 on normal human skin, mouse skin, and salt-split skin substrate. The affinity-purified autoantibodies were also assessed by Western blot analyses and ELISA as described (Gammon *et al.*, 1984, 1990; Woodley, 1990; Chen *et al.*, 1997a, b).

Preparation of F(ab')₂ fragments

F(ab')₂ fragments of affinity-purified EBA IgG autoantibodies were prepared by digestion with pepsin as described (Liu *et al.*, 1995). Undigested IgG and Fc fragments were removed by affinity chromatography using a protein G-Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden). Purified F(ab')₂ fragments migrated as a 100 kDa band under non-reduced SDS-PAGE. The completeness of digestion was assessed by IIF using mouse skin as substrate and F(ab')₂ preparations demonstrated reactivity only with an FITC-labeled goat anti-human-F(ab')₂, but not with an FITC-labeled goat anti-human-Fc secondary antibodies.

Administration of EBA antibodies, animal evaluation, and characterization

SKHl mice were injected intradermally in the middle back of mice with EBA IgG (*n* = 52), normal human control IgG (*n* = 10), or EBA IgG-depleted NC1 reactivity (flow-through fractions from NC1 affinity column) (*n* = 5) once every day for 8 days and observed every day. IgG doses ranged from 2.5 to 100 μg/g body weight/per day. The animals were photographed daily. Skin erythema, blisters, and erosions were recorded. Mice that developed blisters had skin biopsies from the blisters and non-blistered normal-appearing skin within 0.5 cm of a blister. Skin samples were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Both lesional and perilesional tissues were subjected to DIF staining as previously described (Gammon *et al.*, 1990; Woodley *et al.*, 1984). Monospecific FITC-conjugated sera were obtained commercially: goat anti-human IgG (Sigma, St Louis, MO), monospecific goat anti-mouse C3 (Cappel Laboratories, Durham, NC), goat anti-mouse neutrophlis (Cedarlane, Ontario, Canada), goat anti-human F(ab')₂, and Fc (Cappel Laboratories, Durham, NC). Photographs of immunolabeled tissues were obtained with a Zeiss Axioplan fluorescence microscope equipped with a Zeiss Axiocam MRM digital camera system.

Direct immunogold electron microscopy was performed on the lesional and perilesional regions of skin from SKH1 mice injected with affinity-purified EBA antibodies. Following excision from the mouse, these portions were incubated in goat anti-human 1-nm gold conjugate (Amersham Biosciences, Piscataway, NJ) and further processed for transmission electron microscopy as described previously (Rousselle *et al.*, 1991).

Sera were obtained from the mice at the time of the biopsy and examined for anti-type VII collagen antibodies by IIF, Western blot analysis, and ELISA, as described above (Gammon *et al.*, 1984, 1990; Woodley, 1990; Chen *et al.*, 1997a, b). Animals given normal human control IgG or flow-through IgG fractions from NC1 affinity column were studied in an identical fashion.

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